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CpG-oligodeoxynucleotides enhance porcine immunity to *Toxoplasma gondii*

H. Kringel[a], J.P. Dubey[b], E. Beshah[c], R. Hecker[d], J.F. Urban Jr.[c,*]

[a] *Danish Center for Experimental Parasitology, The Royal Veterinary and Agricultural University, Dyrlægevej 100, DK-1870 Frederiksberg C, Denmark*

[b] *United States Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory, Beltsville, MD, USA*

[c] *United States Department of Agriculture, Agricultural Research Service, Beltsville Human Nutrition Research Center, Nutrient Requirements and Functions Laboratory, Building 307, BARC-East, Beltsville, MD 20705-2350, USA*

[d] *Qiagen GmbH, Hilden, Germany*

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Abstract

Protection against a challenge infection with *Toxoplasma gondii* VEG strain oocysts was examined in pigs after vaccination with *T. gondii* RH strain tachyzoites with or without a porcine specific synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs. Six groups of pigs were immunized with incomplete Freund's adjuvant (IFA) and either vehicle, tachyzoites alone or in combination with three different doses of CpG ODN or with CpG ODN alone. Protection from challenge was significantly ($P < 0.05$) improved in pigs vaccinated using CpG ODN as an adjuvant with tachyzoites compared to all other groups. The CpG ODN tachyzoite-immunized pigs also had higher serum parasite specific IgG antibody, no clinical signs of disease, and 52% had no demonstrable tissue cysts after the challenge infection. These data indicate that CpG ODN is a potential safe and effective adjuvant for the *T. gondii* RH strain vaccine in pigs.

Keywords: Synthetic adjuvant; Immunostimulatory sequences; Protective immunity

1. Introduction

Synthetic oligodeoxynucleotides (ODN) containing non-methylated CpG motifs are related to naturally derived bacterial DNA and induce a Th1-derived type 1 immune response

* Corresponding author. Tel.: +1 301 504 5528; fax: +1 301 504 9062.
E-mail address: urbanj@307.bhnrc.usda.gov (J.F. Urban Jr.).

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in mammals (Krieg et al., 1995). This involves the early cytokine production of interleukin (IL)-12, tumor necrosis factor (TNF)-α and interferon (IFN)-γ, a cytokine milieu that promotes Th1 cell differentiation (Halpern et al., 1996; Klinman et al., 1996) and NK cell activity (Chace et al., 1997). Delivery of CpG ODN with a large variety of antigens can augment antigen-specific cell-mediated and humoral immunity (Chu et al., 1997; Roman et al., 1997). It enhances antigen presentation and induces B-cell proliferation, activation, IL-6 secretion, and specific immunoglobulin (Ig) isotype switching to IgG2a in mice (Klinman et al., 1996; Krieg et al., 1995).

Th1-derived immune responses protect against intracellular pathogens. Administering CpG ODN with vaccines against several bacteria, viruses, and parasites can enhance protective responses and increase resistance against lethal challenge by promoting Th1 immunity (Elkins et al., 1999; Krieg et al., 1998; Walker et al., 1999). CpG ODN works in synergy with other adjuvants such as incomplete Freund's adjuvant (IFA) and alum to enhance the humoral response to an antigen above the level of any of the three used alone (Weeratna et al., 2000). *Toxoplasma gondii* is an obligate intracellular protozoan parasite that causes toxoplasmosis in humans and animals. Ingestion of undercooked infected pork is considered a major source of human infection in the United States (Dubey, 1994). Humans and most other mammals become infected by consuming raw or undercooked meat containing *T. gondii* tissue cysts (Dubey, 1998a), by ingesting food and water contaminated with sporulated *T. gondii* oocysts shed in cat feces or by inadvertent oral contact with contaminated soil (Dubey, 1994; Dubey et al., 1995; Dubey, 1998a). Tissue cysts form primarily in the skeletal and heart muscle of pigs, but also in the central nervous system (Dubey et al., 1996). Generally, *T. gondii* causes sub-clinical infection, but can be a danger to immune-suppressed individuals and to the fetus in humans, sheep, goats, and pigs because of trans-placental transmission. Prevention of *T. gondii* infection or reducing the number of viable tissue cysts in pigs, avoiding contact with cat feces, and proper cooking of pork is of great public health importance since no vaccine exists for pigs or humans.

The knowledge of the immune response to *T. gondii* in pigs is limited when compared to what is known from studies in mice, but some observations in rodents and humans can be extrapolated to swine to devise a strategy for immune induction. The natural route of infection with *T. gondii* oocysts or tissue cysts (bradyzoites) is by mouth. The response is normally mild to asymptomatic and associated with the rapidly dividing and invasive tachyzoite stage of the parasite. Exposure is thought to result in lifelong immunity to further infection in immune-competent individuals, but tissue cysts derived from the initial infection are quiescent and can become activated when immunity is suppressed. In the chronic phase, immunity is maintained by an adaptive Th1 mediated response, depending on $CD8^+$ T-cells (Parker et al., 1991), IFN-γ (Scharton-Kersten et al., 1996) and specific antibody production (Kang et al., 2000; Sayles et al., 2000). Observations of lifelong immunity, in principle, increase the likelihood of developing a safe and effective vaccine against *T. gondii*. Pigs vaccinated with tachyzoites of the *T. gondii* RH strain, which fails to produce persistent tissue cysts in swine, remain clinically normal and develop fewer tissue cysts than non-vaccinated pigs when given an oral challenge infection with *T. gondii* oocysts (Dubey et al., 1991, 1994). However, protective immunity depends on vaccination with large doses of RH strain tachyzoites.

The purpose of this study was to evaluate the adjuvant properties of a synthetic CpG ODN, that stimulates porcine lymphocytes in vitro, in combination with a low dose of the *T. gondii* RH strain vaccine and to evaluate the level of resistance to an oral challenge with infective *T. gondii* oocysts.

2. Materials and methods

2.1. Experimental animals and bioassay for T. gondii tissue cysts

Forty-eight Yorkshire X Poland China crossbred pigs were obtained from an experimental herd maintained at the Beltsville Agricultural Research Center. Pigs were 5–6 weeks of age at the start of the experiment. They were housed two per pen in stalls with a non-absorptive concrete floor surface, and had access to water and fed a corn-soybean formulation containing 16% crude protein and vitamins and minerals that exceeded NRC guidelines (Urban et al., 1989) ad libitum.

Tissues of pigs were evaluated for the presence of viable *T. gondii* tissue cysts by semi-quantitative bioassays. Two bioassay systems (mice and cats) were used. Pig tongue and brain was tested in Swiss Webster (SW) mice when 50 g of pig brain and tongue from each pig were homogenized in saline (0.85% NaCl), digested in pepsin for 60 min at 37 °C, neutralized, washed, suspended in saline, and injected sub-cutaneously into 10 mice (Dubey, 1998b). The entire sediment from the tongue homogenate and approximately 1/10 of the brain homogenate were injected into mice. The mice were subsequently examined for *T. gondii* infection (Dubey et al., 1996). Briefly, imprints of lungs of mice that died were examined for *T. gondii* tachyzoites. Survivors were bled 6 weeks later and 1:25 dilution of serum of each mouse tested for *T. gondii* antibodies in the modified agglutination test (MAT) (Dubey and Desmonts, 1987). Mice were killed 1–2 weeks after serologic examination and brain squash of each mouse was examined for tissue cysts (Dubey and Beattie, 1988). Mice were considered infected with *T. gondii* when tachyzoites were demonstrable in their tissues or when positive serum antibody titers exceeded a 1:25 dilution (positive titer).

In the cat bioassay portions of brain, tongue and skeletal muscle from the limbs of infected pigs were pooled and approximately 500 g were fed to cats over a period of 3 days. The cats were *T. gondii*-negative before feeding porcine tissues and were raised in the BARC SPF cat colony. Management of the cat colony, fecal collection and examination for *T. gondii* oocysts has been previously described (Dubey, 1995). The cat bioassay is much more sensitive than the mouse bioassay because the cat is the definitive host and sheds numerous oocysts after ingesting as few as one bradyzoite (Dubey, 2001).

2.2. Vaccine and challenge inoculum

The porcine-unique CpG DNA oligonucleotide (CpG ODN 2007, provided by Qiagen GmbH, Hilden, Germany) was dissolved in endotoxin-free phosphate buffered saline (PBS, pH 7.4, Gibco) to a final concentration of 20 mg/ml (stored at −70 °C). The line of *T. gondii* RH strain used (vaccine) is self-limiting in pigs and becomes undetectable 2 weeks after inoculation (Dubey et al., 1991). Tachyzoites were obtained from exudates of the peritoneum of experimentally infected SW mice, counted in a hemacytometer, and suspended in endotoxin-free PBS to a final concentration of 4000 tachyzoites/ml.

The vaccine for each group was prepared just before injection using two connecting syringes to form an emulsion of incomplete Freund's adjuvant (Difco, Detroit, MI) 1:1 with CpG ODN, PBS and the tachyzoite suspension. Each pig was injected intramuscularly with 1 ml of the vaccine preparation using a 20-gauge needle.

T. gondii VEG strain oocysts used for challenge were obtained from the feces of experimentally infected cats by differential centrifugation and passage through a series of sieves, sporulated at room temperature for 1 week, and stored for 6 months in 2% H_2SO_4 solution at 4 °C. Oocyst suspensions were neutralized with 3.3% NaOH solution immediately before inoculation and the volume adjusted with saline (Dubey et al., 1996). Each pig was orally inoculated using a rounded-end feeding needle. Oocyst infectivity was tested in mice (Dubey et al., 1996).

2.3. Experimental design

Pigs were divided into six different treatment groups with 8 pigs per group. All groups were immunized on day 0 and boosted on day 26 post-immunization (p.i.). Pigs from Group 1 were given IFA containing PBS. Pigs from Groups 2–5 were immunized with 10^3 VEG strain *T. gondii* tachyzoites per pig in IFA and pigs from Groups 3–5 also received in the vaccine emulsion 5, 1 or 0.2 mg CpG ODN per pig, respectively. Pigs from Group 6 received only 5 mg CpG ODN in IFA. All groups were orally challenged with 10^4 *T. gondii* oocysts on day 49 p.i. and euthanized 54–63 days later.

2.4. Evaluation of immune responses

Rectal temperatures were recorded for all pigs after immunization, boost and challenge.

Pigs were bled every 1–2 weeks and serum stored at −20 °C until examined. Serum IgG antibody to whole formalinized-*T. gondii* tachyzoites (parasite specific antibody) was assayed by the MAT (Dubey and Desmonts, 1987) and serum IgG to fixed *T. gondii* tachyzoite antigen were assayed by dual end-point dilution enzyme-linked immunosorbent assay (ELISA) (IVD Laboratories, Carlsbad, CA). In the MAT, sera from individual animals were diluted 1:25, 1:100, 1:500 and 1:5000 and pigs were considered serologically negative with a titer below 1:25. Titers for parasite specific IgG antibody (positive titers) were defined as the highest sample dilution that resulted in an absorbance value ($OD_{450–650}$) equivalent to or higher than two standard deviations above mean of all 1:200 pre-bleed titers. Sera were pooled within each experimental group for the ELISAs.

2.5. Statistical analysis

Group means were evaluated by ANOVA with Tukey's analysis to compare individual groups. Values in the figures are expressed as mean + standard deviation (S.D.). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Clinical signs

All pigs appeared normal and none of the pigs had elevated body temperature after immunization or boost. However, at 5 days after challenge inoculation with oocysts, most pigs from Group 1 and 6 became febrile. The average body temperature of these groups differed significantly ($P < 0.05$) from pigs in the Groups 3–5 that were immunized with tachyzoites and each of the three different doses of CpG ODN (Fig. 1). The mean temperature of pigs from Group 2 that were immunized with tachyzoites alone was elevated, but not significantly different from that of pigs immunized with tachyzoites in combination with CpG ODN. One pig from Group 5 (no. 34) died inexplicably on day 33 p.i.

3.2. The humoral response

All pigs, except for one from Group 4, were serologically negative in the MAT and in the *T. gondii* specific IgG ELISA before *T. gondii* vaccination. A titer increase relative to PBS-vaccinated controls (Group 1) appeared on day 10 and 14 p.i. in all tachyzoite-vaccinated groups, as reported previously (Lind et al., 1997). However, the titer increase was more rapid and reached an approximately 10-fold higher level in pigs from groups that in addition to RH strain tachyzoites also received the CpG ODN. Except for a slight increase in pigs in the low dose CpG ODN (0.2 mg per pig) RH strain vaccinated group, titers remained at the same level after the boost. All antibody titers increased following *T. gondii* oocyst challenge (49 days p.i.). However, the MAT assay indicated a CpG ODN dose-dependent decrease in titer relative to the PBS-vaccinated and CpG ODN alone-vaccinated groups at the time of necropsy (Fig. 2).

3.3. Level of protection

Tachyzoites of *T. gondii* RH strain disseminate in pigs when injected intramuscularly, but disappear without encysting during the second week of infection (Dubey et al., 1994). The *T. gondii* RH strain is lethal for mice within 17 days after inoculation, and therefore, mice dying within this interval after inoculation with tissues from infected pigs would indicate residual RH strain organisms. In contrast, *T. gondii* VEG strain is relatively non-virulent in mice, but will persist in mice and generate an antibody response (Dubey et al., 1999). No mice used in the bioassay died during the 17-day interval after inoculation with pig tissues obtained from any of the groups after the challenge inoculation with VEG strain oocysts, indicating that there were no tissue cysts persisting from the RH strain. This is consistent with earlier studies using 3-log higher doses of RH strain tachyzoites (Dubey et al., 1991). Tissue cysts were found in the lungs and brain of each of the mice that subsequently sero-converted following injection with pig tissues. The percentages of mice that became infected with *T. gondii* when injected with tissue homogenates from any of the groups immunized with tachyzoites plus CpG ODN (Groups 3–5) were significantly lower ($P < 0.05$) than the percentages of mice that became infected when injected with tissues from pigs in the PBS, RH strain alone or CpG ODN alone immunized groups (Table 1).

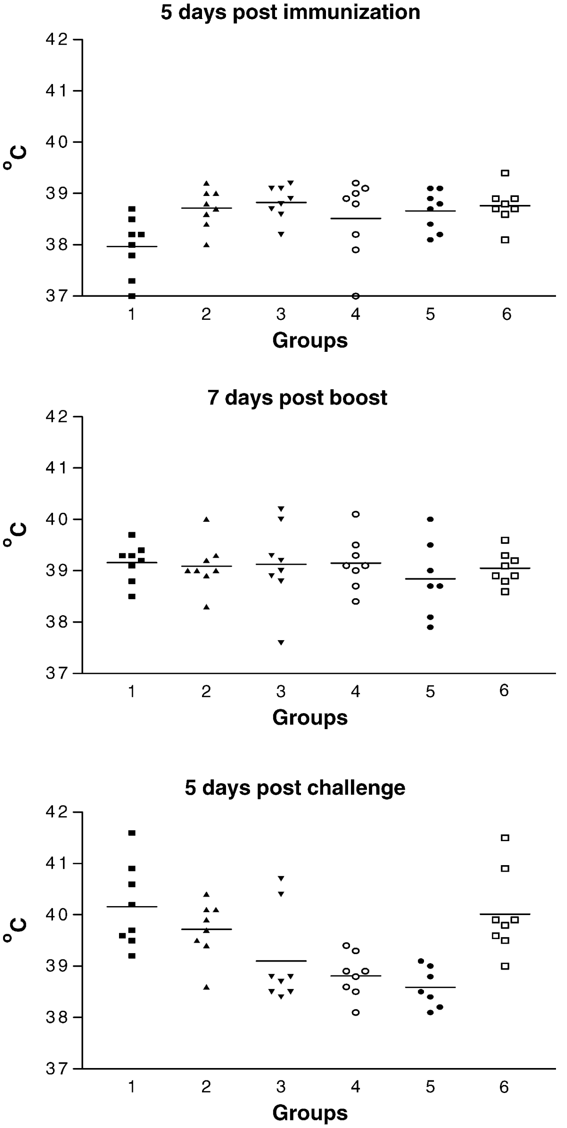

Fig. 1. Rectal temperatures for all pigs recorded 5 days p.i., 7 days post-boost and 5 days post-challenge with *T. gondii* oocysts. All treatment groups were immunized with IFA and either: Group 1: PBS; Group 2: *T. gondii* tachyzoites; Group 3: *T. gondii* tachyzoites + high dose (5 mg) CpG ODN; Group 4: *T. gondii* tachyzoites + medium dose (1 mg) CpG ODN; Group 5: *T. gondii* tachyzoites + low dose (0.2 mg) CpG ODN; Group 6: high dose (5 mg) CpG ODN.

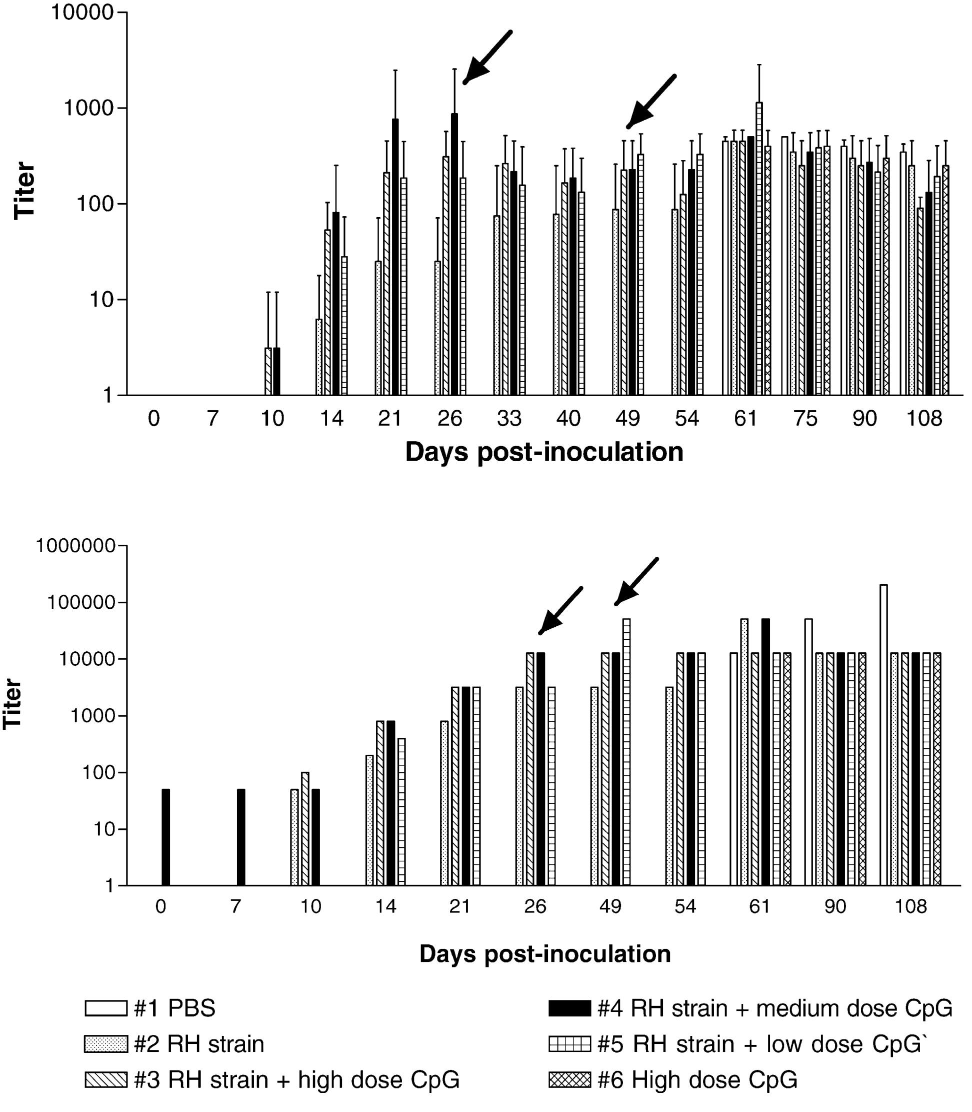

Fig. 2. Top panel: titers for serum antibodies to whole-formalinized *T. gondii* tachyzoites, assayed by the MAT. The error bars indicate S.D. of the mean. Bottom panel: titers for *T. gondii* specific IgG antibodies in serum determined by ELISA. Arrows indicate days of boost and challenge, respectively. Group 1: PBS; Group 2: *T. gondii* tachyzoites; Group 3: *T. gondii* tachyzoites + high dose (5 mg) CpG ODN; Group 4: *T. gondii* tachyzoites + medium dose (1 mg) CpG ODN; Group 5: *T. gondii* tachyzoites + low dose (0.2 mg) CpG ODN; Group 6: high dose (5 mg) CpG ODN.

Results are similar for mice inoculated with brain and tongue tissue. Each of the two cats fed tissues from pigs from Groups 1–3 shed *T. gondii* oocysts. These results were consistent between both the mouse and cat bioassays except for pig no. 23 from Group 3 that gave a positive response in cats but not in mice. Oocysts were not detected in cats fed tissues

Table 1
Bioassay of detectable VEG strain *T. gondii* tissue cysts from oocyst-challenged pigs following vaccination with RH strain *T. gondii* tachyzoites in IFA with or without CpG ODN

Pig no.	In mice		In cats muscle	Infected pigs (%)
	Brain	Tongue		
Group 1				
1	10	10		
2	8	6		
3	10	10		
4	10	10		
5	10	10		
6	10	10	+	
7	10	10		
8	10	10	+	
Mean	9.75	9.50		100
Group 2				
9	10	10		
10	10	10		
11	3	0		
12	2	0		
13	10	10	+	
14	10	10	+	
15	10	10		
16	10	10		
Mean	8.13	7.50		100
Group 3				
17	0	0		
18	0	0		
19	0	0		
20	0	0		
21	1	4		
22	10	10		
23	0	0	+	
24	10	1	+	
Mean	2.63*	1.88*		50*
Group 4				
25	0	0	−	
26	1	0	−	
27	0	0		
28	0	0		
29	7	0		
30	0	0		
31	10	10		
32	0	0		
Mean	2.25*	1.25*		37.5*
Group 5				
33	0	6		
34				

Table 1 (*Continued*)

Pig no.	In mice		In cats muscle	Infected pigs (%)
	Brain	Tongue		
35	0	0	−	
36	0	0	−	
37	0	1		
38	2	10		
39	6	0		
40	0	0		
Mean	1.14*	2.43*		57.1*
Group 6				
41	10	10		
42	10	10		
43	10	10		
44	10	10		
45	10	10		
46	10	10		
47	10	10		
48	8	10		
Mean	9.75	10.00		100

Vaccination protocol for Group 1: PBS; Group 2: RH strain tachyzoites; Group 3: RH strain + high dose CpG; Group 4: RH strain + medium dose CpG; Group 5: RH strain + low dose CpG; Group 6: high dose CpG. Brain or tongue tissue homogenates from each pig were injected into mice and those that were either sero-positive and/or contained tissue cysts in the brain out of 10 tested are shown. Tissues from two randomly selected pigs from Groups 1–5 were bioassayed in cats; results shown as positive (+) when cats shed oocysts or negative (−). The percentage of infected pigs is based on data combined from both the mouse and cat bioassays.

* Mean values which indicate statistical significance ($P < 0.05$).

from pigs in Groups 4 and 5 (Table 1). After combining the results of pig brain and tongue samples that were positive for VEG strain tissue cysts in mice and/or cats, 100% of the pigs in the PBS (Group 1), RH strain (Group 2) and CpG ODN alone (Group 6) immunized groups had detectable infection after challenge. In contrast, only 48% of the pigs vaccinated with RH strain and CpG ODN (Groups 3–5) became infected after challenge (Table 1). CpG ODN immunization alone did not affect protection against oocyst challenge and had levels of infected mice comparable to pigs immunized with PBS.

4. Discussion

Several studies have demonstrated the immune-stimulatory properties of parenterally delivered CpG ODN in protocols used to vaccinate several animal species, but not previously in pigs. A recent study has found CpG ODN to be an effective immune stimulating adjuvant when immunizing pigs with OVA (Van der Stede et al., 2002). Pigs vaccinated with 10^6 *T.*

gondii RH strain tachyzoites remain free of clinical signs of infection and develop fewer tissue cysts after a challenge with *T. gondii* oocysts (Dubey et al., 1991). In the current study, a unique porcine immune-stimulatory CpG ODN was tested in combination with a dose of the *T. gondii* RH strain vaccine 3-logs lower (10^3 versus 10^6 tachyzoites) than previously used without adjuvant (Dubey et al., 1991), and its ability to improve protective immunity was evaluated.

Although cellular immunity is considered the most important part of the immune response to *T. gondii*, antibodies do play a role in limiting its spread because macrophages kill intracellular parasites coated with antibodies (Sibley et al., 1993). Specific antibodies to *T. gondii* persist probably for life and measuring the antibodies is an easy and reliable method to evaluate prior exposure to *T. gondii* in pigs (Dubey et al., 1995). The stronger parasite antigen specific antibody response during the immunizing phase and the absence of fever or a further increase in antibody titer after challenge in pigs vaccinated with the RH strain in combination with CpG ODN suggests that there is reduced or a markedly limited parasite proliferation from the challenge inoculum. This contention is supported by the fact that the serum antibody response did not increase further after a RH strain/CpG ODN vaccination boost indicating reduced antigen shedding or a lack of development of parasites from the inoculum in immune pigs following vaccination.

The pigs that received the CpG ODN in combination with the RH strain tachyzoites seem to have improved immune defenses compared to the pigs vaccinated without CpG ODN or with CpG ODN alone; 52% of these pigs were completely protected against an oral challenge with oocysts. Thus, CpG ODN provides an enhanced adjuvant property that stimulates antibody production in pigs. The mouse bioassay is consistent and reliable, but is only a semi-quantitative measure of detectable tissue cysts and is limited by the relatively small amounts of pig tissue that can be assayed. However, the results of the mouse bioassay were generally confirmed with greater sensitivity using a bioassay in cats fed 10-fold greater amounts of pig tissue with the capability of amplifying a single tissue cyst in the gut of the definitive feline host. Availability of limited numbers of *T. gondii*-free cats precluded their use as a definitive assay for all pigs that were exposed to *T. gondii* oocysts. Nevertheless, some pigs from the CpG ODN and RH strain tachyzoite-immunized groups were completely negative for *T. gondii* cysts indicating a significant positive effect of using CpG ODN as an adjuvant when vaccinating against *T. gondii* in pigs.

There were no apparent toxic effects and no increase in body temperature after immunization with CpG ODN at the doses used. Thus, CpG ODN may be considered a safe yet effective adjuvant in pigs. Other advantages of utilizing CpG ODN as a vaccine adjuvant include its relatively easy and inexpensive synthesis, its stability and the possibility of administration through mucosal routes (Horner et al., 1998; McCluskie and Davis, 1998; Moldoveanu et al., 1998). The host intestine is the natural site of entry of *T. gondii* (Dubey, 1997) and development of a mucosal immune response would limit the spread of infective tachyzoites to edible tissues of the pig. These facts support future work where porcine immune-stimulatory CpG ODN can be used as an effective adjuvant with a defined peptide vaccine or possibly a DNA vaccine against toxoplasmosis that would reduce transmission of *T. gondii* from pigs to humans.

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